Barriers against DNA-loop formation in a porous matrix

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The resistance towards DNA bending imposed by a porous matrix has been investigated by studying the rate of helix-loop penetration through agarose gel pores as driven by electric fields between 1.1 and 6.7 V/cm. YOYO-stained DNA molecules (680 kilo-base-pairs) were prepared in a well-defined globally oriented state by an electrophoretic procedure (YOYO denotes dimer of oxazole-yellow). Loop initiation by a field perpendicular to the global orientation was detected by linear dichroism (LD) spectroscopy in terms of an initial net helix orientation perpendicular to the applied field direction, reflecting the stretching of the chain between the loopholes by the initial growth of the comparatively weakly oriented loop heads. The rate of loop nucleation exhibits a strong field dependence in agreement with a model based on the entropy cost of loop formation. The effect of increasing the average pore radius from 0.7 to 3 P, where P is the persistence length of DNA (500 Å), is significantly weaker than predicted from the model, however. After initially being perpendicular, the net helix orientation is eventually along the field direction, and during this phase the LD exhibits several oscillations before reaching a steady state. By comparison with fluorescence microscopy observations on individual molecules under identical conditions the LD oscillations are identified in terms of loop growth and competition. The spectroscopically measured average rates of these later loop processes exhibit considerably weaker field dependence than loop nucleation, and with power-law dependencies ($E^{1.2-2}$) in agreement with the DNA coils being stretched by electrophoretic transport of the polymer ends. [S1063-651X(96)01312-8]

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I. INTRODUCTION

Formation of loops of double-stranded DNA is important during electrophoresis in porous gels. In the accompanying paper [1] it is shown that field-aligned loops of DNA pulled out of the reptation tube slow down the electrophoretic migration. Loop dynamics have also been suggested to be involved in high-frequency modulated gel electrophoresis [2]. Recently Viovy and co-workers suggested a separation mechanism for DNA in a porous system [3], based on field-driven loop penetration through a set of pores.

Formation of DNA loops is also studied intensely in many biological systems, such as bending of the helix by regulatory proteins [4,5] and in the organization of the DNA in the nucleus [6] and chromosomes [7]. It is therefore important to study how the restricted geometries typical of the cytoplasm and nucleus affect loop formation by DNA. Actually, in later years the large body of data collected by microscopy, spectroscopy, and numerical simulations has brought the studies of DNA in gels to such an advanced level (see [8] for review) that this field provides one of the best model systems for detailed studies of polymer dynamics in restricted media.

Here we use agarose gels with a range of average pore radii that straddles the persistence length of DNA, in order to study how the rate of loop formation in a restricted geometry is affected by DNA stiffness. Using an electric field, the force employed to pull the loops through the pores can be tuned to the energy cost of the loop formation, permitting sensitive probing of the barrier provided by the DNA resistance to bending and loop entropy. The basic approach is to use migration in an electric field to prepare DNA molecules that are globally aligned in one direction, but give the DNA molecules enough field-free time to relax all tension built into the chain as a result of the transient anchoring of the molecules around gel fibers during migration [8]. Linear dichroism and microscopy are then used to study the creation and growth of loops of DNA as a perpendicular electric field is applied. A similar initial state of locally relaxed DNA in field-aligned tubes was used by Gurrieri *et al.* [9] in a microscopy study of how *T*2-DNA reorients in pulsed field electrophoresis. In this study we use linear dichroism (LD) spectroscopy to study the kinetics of the loop initiation, which cannot be studied by microscopy because of the limited spatial resolution. Microscopy is used to discriminate between possible interpretations of the spectroscopic averages, in particular regarding the subsequent stages of loop growth and competition. In order to facilitate the combined use of LD and microscopy data, all experiments in this study have been performed with YOYO-stained DNA (YOYO denotes dimer of oxazole-yellow). However, comparison of the field-free decay of the electrophoretic orientation of YOYO-stained DNA with that of native DNA reported in the accompanying paper [1] shows that the results presented here are relevant also for native DNA.

II. MATERIALS AND METHODS

DNA and gel samples

All experiments have been performed in 0.5× TBE buffer (50 m*M* Tris, 50 m*M* borate, 1.25 m*M* EDTA, *p*H=8.2) at 20 °C. DNA from *G* [740 kilo-base-pairs (kbp)] and *T*2 (164 kbp) phages were prepared in gel plugs (about 8 μg DNA in a 6×3 mm agarose cylinder) as described previously [10]. The DNA was stained by immersing each plug in 200 μl of

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1.7 μM YOYO-1 (Molecular probes) in electrophoresis buffer for at least 4 d, at room temperature in the dark to avoid photodegradation [11]. Gels were 0.5%, 1%, or 2% DNA-grade (Biorad) or 2% nusieve (FMC) agarose, with the average pore radii being 1400, 890, 570 [12], and 380 Å [1], respectively. Some experiments were performed in 0.2% agarose, which formed a viscous solution instead of a macroscopic gel.

Linear dichroism

Here we use linear dichroism [1,2] to measure the degree of helix orientation of DNA molecules in terms of an orientation factor S [1]:

$$S = \frac{3\langle \cos^2 \theta \rangle - 1}{2}, \tag{1}$$

where θ is the angle between the helix axis and a reference field direction (the probe field, see below). S ranges between 1 and -0.5 corresponding to perfect helix orientation parallel and perpendicular to the field direction, respectively [13,14]. $S=0$ occurs for randomly oriented DNA molecules but may also result from cancellation between LD contributions from perpendicularly oriented parts within each molecule [14]. In this study we only exploit the time dependence of the LD responses, in order to obtain information on DNA orientation kinetics as the molecules are subjected to changes in the electric field. The LD (at each gel concentration) is therefore presented in terms of S normalized to the steady-state values S_{SS} in a constant field (6.7 V/cm if not otherwise stated)

$$S^n(t) = S(t)/S_{SS}. \tag{2}$$

S^n retains the sign information, in such a way that $S^n<0$ indicates a net orientation of the helix axis perpendicularly to the (probe) field direction. The LD responses observed here reflect helix-orientation effects (and not changes in secondary structure [13]) since DNA remains in the B form during gel migration in orthogonally pulsed fields [14].

LD experiments (at 490 nm) have been performed in a horizontal electrophoresis cell that allows the field to be applied in orthogonal directions in the horizontal plane [14]. A small fraction of degraded DNA in the sample plugs (present before YOYO staining) was separated from the intact DNA by field-inversion gel electrophoresis (1% agarose FIGE; 7.5 V/cm $T^+=40$ s, $T^-=13$ s for G-DNA, $T^+=3$ s, $T^-=1.5$ for $T2$-DNA). The zone of intact DNA was transferred into the measuring position in the attached sample gel (of desired composition) by constant field electrophoresis (3 V/cm for 30 min). During separation and transfer some YOYO dye will be lost [15], so care was taken to follow this standard protocol. The final DNA concentration in the measuring position was approximately 0.075 mM DNA phosphate.

Fluorescence microscopy

A previously described fluorescence microscopy system [16] was used, employing the following protocol for DNA purification and microgel preparation. The FIGE purification of the YOYO-stained DNA was performed in low-melt agarose (instead of normal agarose) and the cutout DNA zone was remelted (65 °C for 5 min), and diluted 30-fold with 1% low-melt agarose in argon-saturated electrophoresis buffer containing 5% mercaptoethanol. From solidified gel blocks containing the diluted and YOYO-stained DNA, gel slices approximately $5\times2\times1$ mm^3 were cut out and put in the 1-mm-deep sample compartment of a specially designed electrophoresis cell for microscopy (Fig. 1). The compartment was closed with a coverslip (which was in contact with the top surface of the gel slice) and filled with agarose solution of the desired composition (again argon bubbled and with 5% mercaptoethanol). DNA was finally transferred into the surrounding sample gel by electrophoresis (30 min, 3 V/cm), thus mimicking the preparation steps of the LD experiment, the only difference being the remelting of the FIGE gel. That the molecules remained intact during this procedure was strongly indicated by the small fluctuations between different molecules in the apparent length measured in the most extended (U formed) state in the reorientation process that is studied in this work. (See Table II in the Results section.)

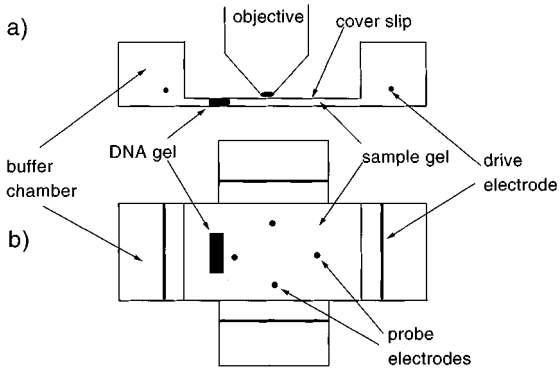

FIG. 1. Electrophoresis cell for microscopy. Four buffer chambers with platinuum wire drive electrodes (pairwise providing two perpendicular fields) are connected via a 0.1-cm-thick sample gel formed under a coverslip. (a) Side view, with one pair of buffer chambers absent. (b) Top view. Platinuum point electrodes for field measurements (in contact with the sample gel from below) and position of gel piece originally containing the DNA are indicated. With this arrangement the drive electrodes were removed far enough from the observation area (inside the probe electrodes) that the two field directions were perpendicular without isolation of the nonactive electrode pair.

From the strong binding of YOYO to DNA [17] it can be estimated that with the employed staining protocol the DNA molecules are saturated with bisintercalated dye, and in addition contain some externally bound dye. It was found that the preelectrophoresis had the advantageous effect of reducing DNA photocleavage, without leading to any significant reduction of the brightness of the imaged molecules. This observation is consistent with loss of externally bound dye, which is less strongly bound and less fluorescent [17] but photochemically more active [11] than intercalated dye. Thus, although the exact binding ratio is not known, the amount of bound dye in the LD and microscopy experiments will be similar, and corresponding to nearly saturation with respect to intercalated dye but essentially no externally bound dye. As a result the molecules will be 50% longer (G,

320 μm; $T2$, 79 μm) and have 15% lower charge than native DNA [18] while results presented below indicate that the effect of YOYO on the DNA persistence length is small.

The reptation model in LD and microscopy

Within the reptation model, the conformation of a DNA molecule in a gel is described at two levels. The global path of the molecule between the gel obstacles (the primitive path) is represented by a fictitious tube, and secondly the conformation of the DNA inside the tube has to specified. The fluorescence microscopy image of a DNA molecule in a 1% agarose gel essentially corresponds to the reptation tube filled with a DNA molecule with a non-resolved intratube conformation [16]. In LD there is no corresponding limitation due to spatial resolution, so for reptating DNA (see [1] for LD theory) the orientation factor S factorizes into two contributions:

$$S = S_{\text{tube}} S_{\text{local}}, \tag{3a}$$

with

$$S_{\text{tube}} = \frac{3\langle \cos^2 \delta \rangle - 1}{2}, \qquad S_{\text{local}} = \frac{3\langle \cos^2 \beta \rangle - 1}{2}, \tag{3b}$$

where δ is the angle between the local reptation-tube axis and the field direction, and β the angle between the DNA helix axis and the local tube axis. S_{tube} thus quantifies the degree of tube orientation and S_{local} reflects the degree of stretching of the DNA along the tube, and both may change when the electric field is altered. In particular, when the field is turned off the degree of stretching decreases, as the chain retracts in response to the tension, and the tube orientation decreases as the DNA diffuses out of the oriented tube. Both processes reduce the helix orientation, but as described in the accompanying paper [1] the two components can be deconvoluted for long DNA on the basis of the large difference in rate. This fact is exploited here to prepare DNA in a state appropriate for systematic studies of loop formation.

Preparation of the initial state

Loop creation is studied by applying a (probe) field to DNA molecules that reside in tubes aligned perpendicular to the field, but that are in a well-defined locally relaxed state without overstretching (see Fig. 2). This standard initial state is created by first orienting the DNA molecules by migration in a preparatory field of 6.7 V/cm, and then turn off the field for a time that is long enough to allow the chain to retract inside the tube (and make it shorter), but short enough to avoid any appreciable degradation of the tube orientation by reptation. The field-off time was determined to be 10 min by direct measurements of the field-free decay of the length of the μ path of the DNA molecules in the gel, and the appropriateness of this time was confirmed by LD experiments (see results).

In the LD experiments the preparatory field was turned off when the LD reached a steady state, which corresponds to a constant average helix orientation in the sample, but with a broad distribution of molecular conformations with different degrees of stretching [16]. In the microscopy experiments the preparatory field was usually turned off when the studied molecule was stretched into an I-shaped conformation by the migration, instead of sampling the whole distribution. This procedure is equivalent to the LD protocol, however, since microscopy showed that even if the field was turned off when the molecule was hooked around a gel fiber in a U conformation, the molecule had relaxed into an I-shaped tube conformation after 10 min. Finally, in both LD and microscopy the perpendicular probe field (of desired strength) is applied to the molecules in this standard initial state. The probe field strength was always lower than that of the preparatory field in order to avoid trapping of the DNA. Check experiments showed that no trapping occurs at 6.7 V/cm in any of the gels studied here, but that after 15 min approximately 50% is trapped at 16 V/cm in all the gels studied.

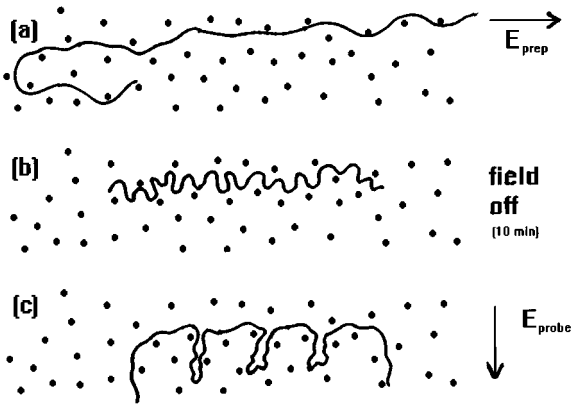

FIG. 2. Schematic presentation of the standard experiment. (a) Alignment of the molecules by electrophoresis. (b) Preparation of locally relaxed molecules by field-free relaxation of stretching. (c) Application of perpendicular probe field.

III. RESULTS

Effect of the waiting time on the LD response to a perpendicular field

Figure 3 shows how the LD response of G-DNA in 0.5% agarose to a probe field of 5.9 V/cm depends on the duration of the field-free period since the orthogonal preparatory field was turned off. For a given waiting time (e.g., 10 min, see inset) the LD buildup is strongly oscillatory with a main overshoot (at t_2), an undershoot (at t_3), and at least one secondary overshoot (at t_4). In addition there is a kink (at t_1) before the first overshoot, giving rise to a marked shoulder in the LD buildup. The LD responses at the other gel concentrations studied are similar in shape, but for a given waiting time the amplitude of the oscillations decreases consistently as the pores of the gel become smaller. Also in the 0.2% agarose solution the DNA molecules respond with an oscillatory LD, in spite of the lack of a permanent network (results not shown). Table I summarizes the characteristic times of the LD responses in 1% and 0.5% agarose gels for a waiting time of 10 min.

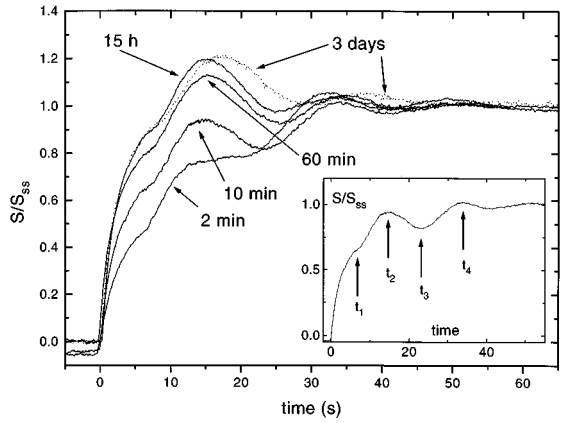

FIG. 3. Orientation response of *G*-DNA to a probe field of 5.9 V/cm (applied at time zero) after different duration of the field-free period (indicated) after the preparatory field was turned off. The orientation factor S is normalized to its steady state value S_{SS}. Agarose gel concentration 0.5%. Inset: definition of characteristic times in the LD response.

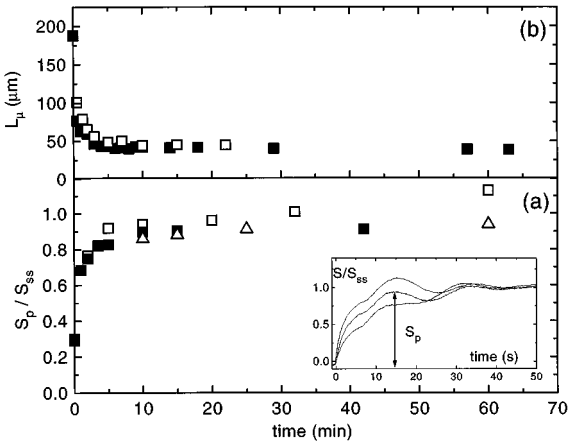

FIG. 4. Determination of the waiting time for the standard initial state. (a) Amplitude of primary orientation overshoot (S_p from LD, see inset) of *G*-DNA and (b) apparent molecular length in the gel (L_μ, from microscopy), vs time of field-free relaxation after the preparatory field was turned off (at time zero). For L_μ, data from two experiments on the same molecule in 1% agarose gel are presented, while S_p data are in 0.5% (□), 1% (■), and 2% (△) agarose gel, respectively. Field strength is 5.9 V/cm.

The main overshoot grows in amplitude as the field-free waiting time is increased from 2 min to 15 h, and there is concomitant shift of the position of the peak (and the shoulder) towards longer times (Fig. 3). The peak position continues to shift for at least 3 d (although there is no further increase in the amplitude), which reflects the very slow equilibration of long polymers in a porous matrix. The slow relaxation is also evidenced by the observation that even after 60 min waiting time there is a weakly negative S before $t=0$, which reflects that some of the helix orientation induced by the preparatory field remains when the probe field is applied.

Preparation of the initial state

In this work the amplitude S_p of the first overshoot [Fig. 4(a), inset] has been used to follow the relaxation of the molecules, in order to determine the relevant waiting time for the loop creation experiments. Initially S_p grows rapidly as the duration of the field-free period is increased [Fig. 4(a)]. After about 6 min, however, the amplitude saturates at a level that is constant up to a waiting time of about 60 min, after which the amplitude starts to grow again (not shown). Thus, in terms of the LD response to the probe field there is a long-lived intermediate state, which furthermore is present in all gels studied here since similar results are obtained at the other gel concentrations [Fig. 4(a)].

TABLE I. Response times to probe field from LD. *G*-DNA in standard initial state (see text). Field strength 5.9 V/cm. Agarose concentration indicated.

	1%	0.5%
t_1 (s)[a]	19.5	10.5
t_2 (s)[b]	41.1	23.9
t_3 (s)[c]	58.2	37.5
t_4 (s)[d]	92	54.2

[a]Time to kink.
[b]Time to primary overshoot.
[c]Time to undershoot.
[d]Time to secondary overshoot. (See Fig. 3 for definition of LD times.)

The results in Fig. 4(b) show that the intermediate state indeed is a locally relaxed DNA molecule in a field-oriented tube, at least in 1% agarose. Initially the apparent length of the path (L_μ) decreases very rapidly as the molecule contracts, but also L_μ levels out after about 6 min and stays essentially constant for at least 60 min. During this time span the molecule remains in the original field-aligned tube in the gel, and only after much longer times is there a significant degradation of the tube orientation by reptation (result not shown). No quantitative studies of the field-free decay of L_μ were performed at other gel concentrations, but the picture of a long-lived state of constant L_μ at the other gel concentrations was strongly supported by inspection of the images of DNA molecules in these gels. It is thus concluded that between 6 and 60 min waiting time there is an intermediate state of locally relaxed DNA in a field-aligned tube in all the gels used in this study. In the 0.2% agarose solution the LD overshoot amplitude does not exhibit the same distinct intermediate threshold for intermediate waiting times. The shape of the LD decay indicates that the orientation relaxation is not as markedly biphasic as in gels (result not shown). The nature of the initial state of the DNA in this system is thus less clear, and was not investigated further.

Microscopy studies of loop formation from the standard initial state

Figure 5 shows microscopy images of a typical response of a *G*-DNA molecule in the standard initial state, to the same probe field of 5.9 V/cm as in Fig. 3. Initially a large (>10) number of hernias are formed, but already after a couple of seconds they are reduced to less than typically five. All hernias except one are consumed over the next 10 s or so, at different rates depending on their relative sizes. (The time at which the second to last hernia is consumed is called t_1.)

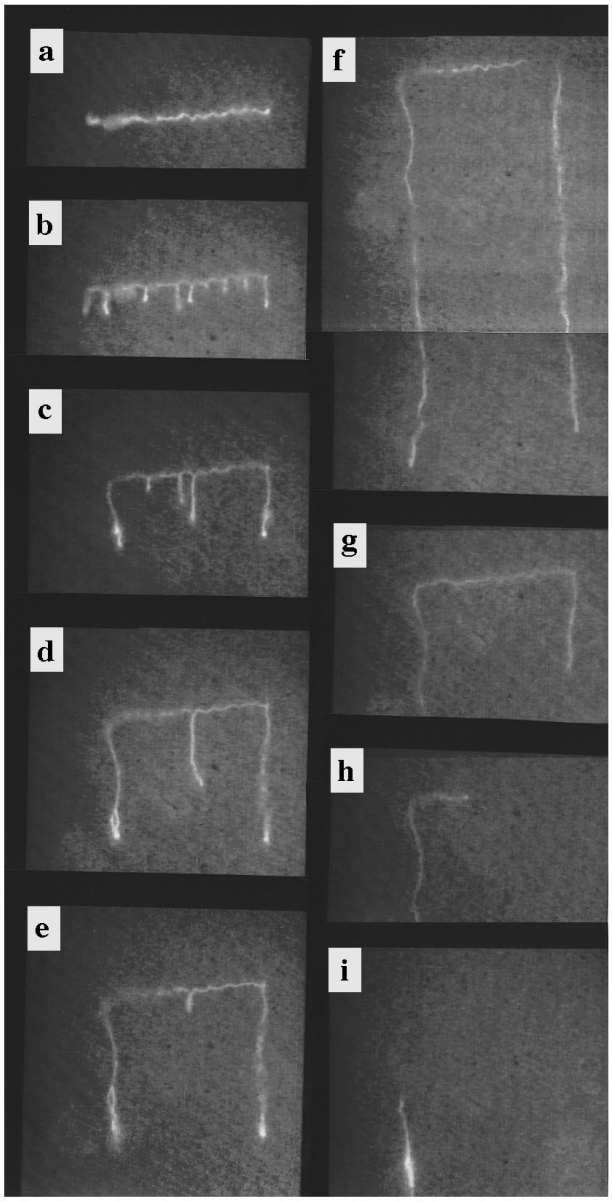

FIG. 5. Fluorescence microscopy images of the response of a *G*-DNA molecule in the standard initial state, to a probe field (vertical) of 5.9 V/cm. (a) Initial state before probe field is applied (at time zero). (b)–(i) Subsequent conformations after 1, 4, 8, 10, 30, 36, 40, and 50 s. The field of view is the same in (a)–(f), but image (f) is a composite picture with a new field of view further downfield. For (g) and (h) the field of view was changed back upfield (only the base of the U is shown), and again downfield for (i), which shows the compact conformation that forms as the trailing end catches up with the head of the winning left arm. The width of the presented field of view is 80 μm. Gel concentration 0.5% agarose.

In most cases the final loop is also consumed (at $t=t_{\mathrm{III}}$, after starting to retract at $t=t_{\mathrm{II}}$), and the corresponding amount of chain is transferred to the arms of the U now formed by the ends of the DNA molecule. However, in about 20% of the cases (4 out of 22 in 1% agarose) the last hernia is long enough to compete successfully and consume one of the arms formed by the ends, and the molecule forms a more narrow U. In any case, there is eventually a competition between the arms of the U, which starts to slide (at $t=t_{\mathrm{IV}}$) with the trailing end following the path of the U. Strikingly often the U is metastable, in the sense that no end makes progress through the gel for many seconds, and it is clear that the chosen standard initial state tends to give a high fraction of nearly symmetric U forms. By contrast, in the comparatively few cases where one of the arms is formed out of the last loop, metastable U conformations are rarely observed.

Finally the trailing end slips around the last corner post (at $t=t_{\mathrm{V}}$), and the molecule collapses into what is often a comparably compact state (at $t=t_{\mathrm{VI}}$). (This is because the winning end by now has formed a highly branched structure, which makes much slower progress through the gel than the trailing end does.) As a result, there is initially a comparably high degree of coherence between different molecules in the subsequent phases where a narrow U is formed out of the compact state and slips into a new compact state, although these steps have not been studied quantitatively.

TABLE II. Response times to probe field from microscopy. *G*-DNA in standard initial state (see text). Probe field 5.9 V/cm. Agarose concentration indicated. s.d. denotes standard deviation.

	1% agarose[a]		0.5% agarose[b]	
	Mean±s.d.[c]	s.d./mean	mean±s.d.[c]	s.d./mean
t_{I} (s)[d]	9.8± 1.2	0.13	8.0± 1.7	0.21
t_{II} (s)[e]	14.3± 2.1	0.15	10.2± 1.8	0.18
t_{III} (s)[f]	18.9± 4.2	0.22	13.9± 3.2	0.23
t_{IV} (s)[g]	38.1± 7.0	0.18	29.1± 3.6	0.12
t_{V} (s)[h]	55.8± 7.6	0.13	42.0± 6.3	0.15
t_{VI} (s)[i]	64.7± 7.9	0.12	51.0± 6.7	0.13
L_0 (μm)[j]	42.4± 6.1	0.14	31.7± 7.8	0.25
$L_{\max}$ (μm)[k]	188 ±15.3	0.08	216 ±20.6	0.10

[a]Nine observations on three molecules.
[b]15 observations on two molecules.
[c]Mean±standard deviation.
[d]Time to death of second to last loop.
[e]Time to start of retraction of last loop.
[f]Time to death of last loop.
[g]Time to start of slipping of the U.
[h]Time to trailing end slipping around last corner of the U.
[i]Time to trailing end catching up with the head.
[j]Apparent length of molecules before probe field is applied.
[k]Maximum apparent length of the molecule in the U conformation.

In Table II the average values and standard deviations of the characteristic times t_{I}–t_{VI} are presented for 1% and 0.5% gels at 5.9 V/cm. The relative widths are considerably narrower than the value of about 0.5 observed for the period of time of steadily migrating *G*-DNA molecules [16], which demonstrates the increased coherence in the response of molecules prepared to have a similar starting conformation [19]. For the case of 0.5% agarose the relative values of the standard deviation (s.d./mean) leveled out after about ten observations (results not shown), so the standard deviations most likely reflect the inherent width of the distributions in the measured time parameters, and not that the number of observations are too few (which may have been the case in the 1% gel with nine observations). It is therefore probably significant that the relative widths are larger for the initial steps (involving the smaller loops) than for the subsequent stages

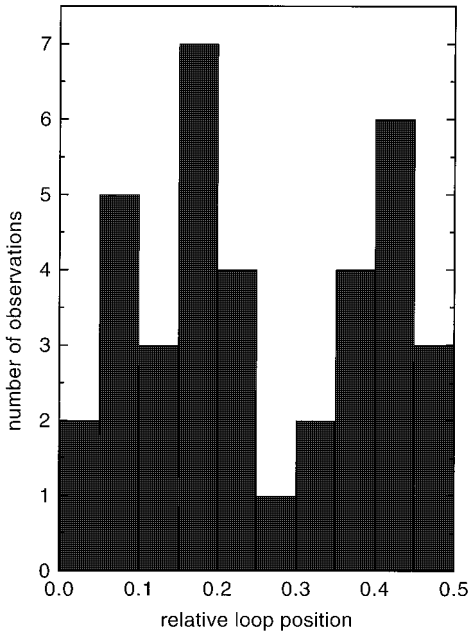

FIG. 6. Histogram of the relative position of the last surviving loop along the base of the U (evaluated from microscopy pictures such as in Fig. 5). The relative position is defined as the absolute value of the difference in coordinate (along the base of the U) for the midpoint and the position of the loop, divided by the total length of the base of the U. A relative position of zero thus corresponds to a loop in the middle of the U, and 0.5 to either end of the base of the U. There are a total of 37 observations on five molecules. Bin size is 0.05. *G*-DNA in 1% agarose gel. Field strength 5.9 V/cm.

of U formation and slippage (0.5% in Table II). This is consistent with the qualitative observation of an initially wide distribution of loop lengths, leading to the process of initial loop growth and competition being more different between different molecules then the later stages of U formation and slipping.

The fact that the ends drive the final steps of the reorientation in a majority of the cases may be of importance for the understanding of pulsed field electrophoresis of mega base pair DNA, but is not a main issue of this study. However, the degree to which the ends control the initial stages of the loop dynamics has implications for the interpretation of the LD results. If the whole process of loop growth and decay was dictated by the growth of the arms, the loops should be consumed systematically from the ends of the original tube towards the middle, and the final loop should be found preferentially in the middle of the base of the U. Figure 6 shows the distribution of the relative position of the last loop (normalized to the total width of the U at the base). The data are rather scattered but it is clear that there is no strong tendency for the last loop to be preferentially towards the middle (relative position of 0). This shows that the loop dynamics is not under total control by the ends.

The microscopy data presented here will be employed below to assign the different phases of the LD responses (Fig. 3). This knowledge will be helpful in the interpretation of the effect of field strength on loop formation and growth, which is the main issue of this study.

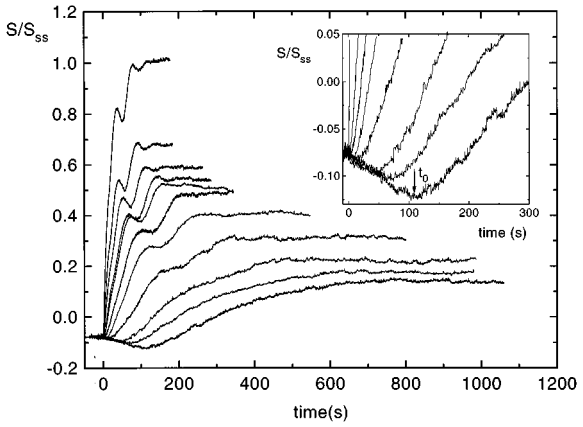

FIG. 7. LD response of *G*-DNA in standard initial state to probe fields of different strengths, in terms of the orientation factor *S* normalized to the steady state value of *S* (S_{SS}) at the highest field strength of 6.7 V/cm. The responses correspond (in order from the highest to the lowest amplitude) to probe field strengths 6.7, 5.7, 4.8, 3.9, 3.4, 3.0, 2.4, 2.0, 1.5, 1.3, and 1.1 V/cm. Inset shows initial part of the responses at (from left to right) 6.7, 3.4, 3.0, 2.4, 2.0, 1.5, 1.3, and 1.1 V/cm, and definition of loop nucleation time t_0 (see text). Agarose concentration is 1%. Note that the normalized *S* before the application of the probe field (at time zero) is the same in all cases because *S* at all fields is normalized to the same value of S_{SS}.

LD responses at different probe field strengths

Figure 7 shows the LD response of *G*-DNA in the standard initial state in 1% agarose to probe fields of different strengths. As the field decreases the orientation buildup is slower, but the oscillatory shape of the response is retained for intermediate fields. However, at even lower fields a new negative LD component evidences that the probe field initially gives rise to a net orientation of the helix, which surprisingly enough is perpendicular to the applied field. The inset of Fig. 7 shows that this initial LD dip is completely absent at high fields, where the LD turns positive immediately, but that it consistently becomes larger in amplitude and slower in rate of growth as the field is decreased, although at intermediate fields it can be observed only as an apparent lag. The time t_0 at which the LD starts to grow (i.e., the position of the minimum and the end of the lag, respectively) will be employed below in the analysis of loop initiation, by invoking the assignment of the subsequent LD phases (after the LD dip) in terms of loop growth and competition.

For a given field strength there is a clear trend for the LD dip to occur later the higher the gel concentration. At 1.1 V/cm (Fig. 8) the dip is clearly visible in 2% nusieve and in 2% and 1% agarose, but only as a delay in the LD growth in 0.5% agarose at this field. In the 0.2% agarose not even a delay is observed, and this holds at all field strengths investigated.

During the set of experiments required to collect a series of LD responses at different fields as in Fig. 7, the molecules of the sample are subjected to a integrated time of electrophoresis corresponding to typically 20 min at 6 V/cm. Potential loss of YOYO with time would affect the LD responses since it is known that the increase in DNA length and reduc-

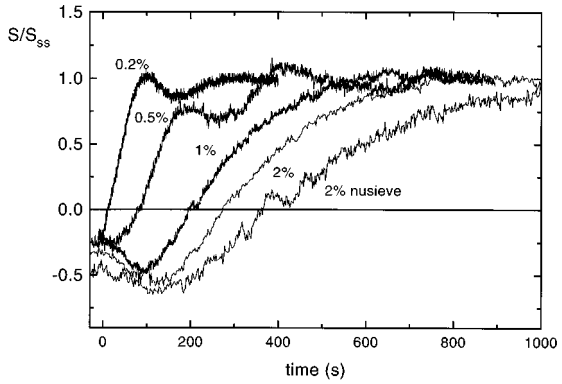

FIG. 8. LD response of *G*-DNA in standard initial state to a probe field of 1.1 V/cm, in terms of the orientation factor *S*, normalized to the steady-state value of *S* (S_{SS}) at the applied probe field strength in respective gel (gel conditions indicated). The value of the normalized *S* before the probe field is applied (at time zero) reflects differences between the gels with respect to remaining orientation after 10 min of field-free relaxation, and to the degree of steady state orientation S_{SS}.

tion of DNA charge by bound YOYO affects the electrophoretic orientation [18]. Such effects were negligible with the present protocol, however, as evidenced by the variations in the LD response in test experiments (at 6 V/cm) inserted at different times during such a series being small compared to the effects of changes in the field strength (results not shown). This observation is consistent with the long effective lifetime of the DNA/YOYO complex during electrophoresis in agarose gels [15]. In fact, if anything, there was a weak trend towards slower responses later in the series, in contrast to the faster responses expected from shorter and more strongly charged DNA molecules that would result from loss of YOYO.

Field dependence in the LD response times

A decrease in the field strength slows down the buildup of the new LD (Fig. 7), and Fig. 9 shows that in a log-log diagram all LD response times (t_0–t_4) exhibit approximately linear field dependencies at all gel concentrations. At a given gel concentration, the exponents of the corresponding potential laws (i.e., the slopes) are very similar for the LD time constants t_1–t_4. By contrast, the time t_0 to the LD dip exhibits a slope that is significantly steeper than for the other time constants in all gels studied.

The slopes for the time constants t_1–t_4 are 1.3 ± 0.1 in 0.5% and 1% agarose, and 2 ± 0.1 in 2% agarose and nusieve, but the details of the behavior of the slopes will be considered elsewhere [20]. The only conclusion drawn here is that these values are consistent with deformation of the DNA driven by the ends of the molecule, since similar slopes are observed for the LD response times of shorter DNA [10,1], where they reflect the rate of coil deformation by electrophoretic transport of the ends [8]. The most interesting piece of information is that the time to reach the onset of LD growth (t_0) is significantly more field dependent, indicating that the field strength affects the rate of the earliest stages of loop formation by a mechanism that is different compared to subsequent loop growth and competition. Figure 10 shows that t_0 is sensitive to pore size, since the curves showing the rapid growth of t_0 with field are consistently shifted towards higher fields as the pore size decreases.

Effect of YOYO on *T*2 relaxation

Since the average pore size in the gels used here is similar to the persistence length of native DNA, it is clear that knowing the bending properties of the DNA will be critical for the understanding of the loop dynamics. The presence of saturating levels of bisintercalated YOYO has a small effect

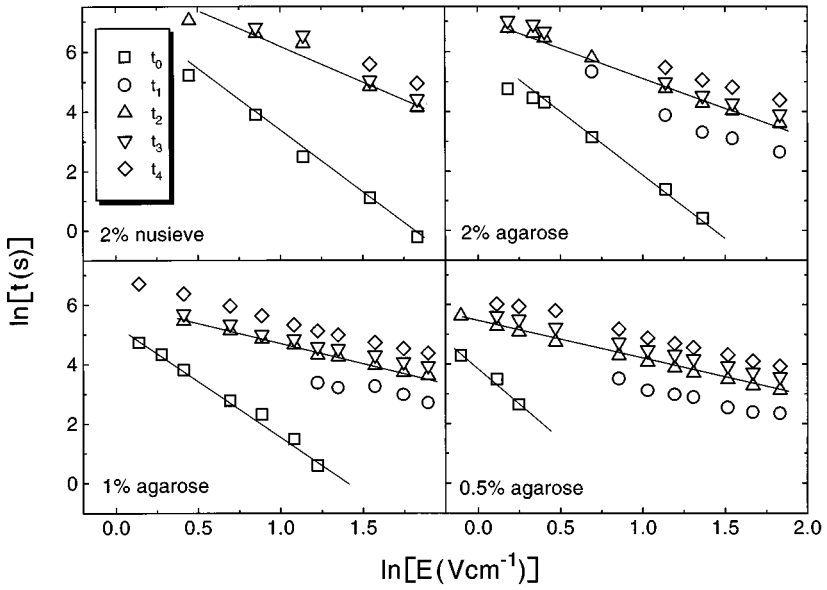

FIG. 9. Log-log plots of characteristic times of the LD response (see legend for symbols and insets of Figs. 3 and 7 for definition) of *G*-DNA in the standard initial state, vs field strength. Gel conditions indicated. Best linear fits (lines) gave the following value of the slopes for t_0 and t_2, respectively: 2% nusieve, −3.93 and −2.18; 2% agarose, −3.80 and −2.04; 1% agarose, −3.67 and −1.28; 0.5% agarose, −4.58 and −1.28.

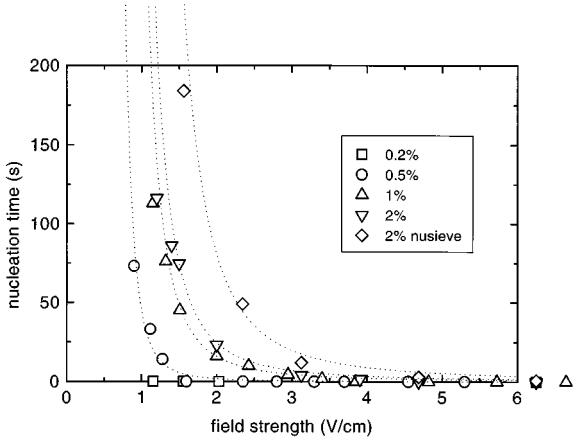

FIG. 10. Loop nucleation time t_0 (inset Fig. 7) vs field strength at different gel conditions (indicated). *G*-DNA. Curves show best fits of Eq. (8). Data from Fig. 9.

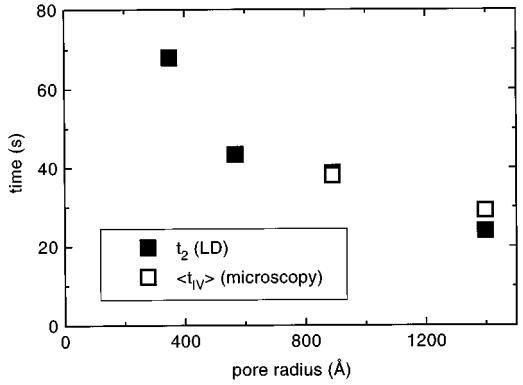

FIG. 12. Time t_2 to LD overshoot (Fig. 3, inset) and average of the time t_{IV} to the formation of a U (Table II) for *G*-DNA in the standard initial state, vs pore radius. (Corresponding to, in order from small to large pore radius, 2% nusieve, 2%, 1%, and 0.5% agarose gels, respectively.) *G*-DNA. Field strength 5.9 V/cm.

on the persistence length of DNA, however. This can be seen from the orientation relaxation of YOYO-stained *T*2-DNA being very similar to native DNA in 2% nusieve gel (Fig. 11), also in the intermediate time range where the relaxation rate is known to be sensitive to the ratio of persistence length to pore size [1]. In fact, the relaxation is somewhat faster with YOYO, in spite of the molecule being longer (the charge reduction of YOYO should have no direct effect on the field-free decay), suggesting that the persistence length decreases if anything. A drastic decrease in persistence length due to a reduction of the electrostatic contribution to the DNA stiffness has been observed as a consequence of partial neutralization of the phosphates by the cationic groovebinder DAPI [21]. A similar effect is expected with the four-valent YOYO, but a counteracting stiffening contribution from the unwinding with YOYO may explain the small net effect on *P*.

IV. DISCUSSION

Conditions for the preparation of the initial state

The LD and microscopy results in Fig. 4 show that the desired initial state of locally relaxed DNA molecules in field-aligned tubes can be accomplished by waiting times between 6 and 60 min. Any time in this range is suitable, since the LD responses that form the experimental basis for the present study do not change in either amplitude (Fig. 4) or characteristic times (Fig. 3) in this time window. A waiting time of 10 min was chosen to define the standard initial state in order to ensure that the intended locally relaxed state was reached, without having unnecessarily long duration of the experiments.

The origin of the different phases of the LD response from the standard state (10 mins in Fig. 3) can now be identified by comparing the characteristic times of the LD signal (Table I) with the average values for time of occurrence of the characteristic reorientation stages identified by microscopy (Table II), since both types of data have been collected for molecules in the standard initial state.

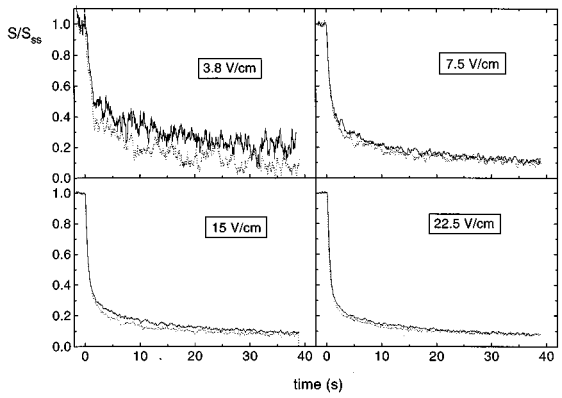

FIG. 11. Field-free relaxation from steady-state LD of native (solid curve) and YOYO-stained (dotted curve) *T*2-DNA, in 2% nusieve gel at different field strengths (indicated).

The primary LD overshoot corresponds to U formation

Both in 0.5% and 1% there is good agreement between the time to the first LD overshoot and the formation of the stretched U, and clearly the second overshoot occurs far too late to correspond to this stage. The overshoot can thus be used to monitor U formation, and Fig. 12 shows that in the limited range investigated by both techniques the averages from microscopy follow well the trend of U creation, becoming slower with increasing gel concentration. A similar agreement at different gel concentrations between the period time of the cyclic migration and the time to the LD undershoot has been reported for *T*2-DNA [8].

In view of these observations it was expected that the first undershoot should correspond to the collapse of the molecule into a compact state after U slipping. However, in 1% agarose the first undershoot occurs between the slip and collapse stages, and in fact in 0.5% it occurs even somewhat before the molecule starts to slip. Thus, in neither of the cases does the undershoot correspond to the collapsed stage. In view of the comparatively high degree of coherence in the reorientation behavior (Table II), the lack of agreement between the LD undershoot time and the collapse time is not likely to be due to a lack of a coherent approach of this state by the ensemble. Instead the disagreement suggests that a large

fraction of the DNA is accumulated in the head (where it has comparatively low orientation) already at the stage when the molecule slips, in agreement with the observation of a highly branched head.

The LD kink corresponds to the death of the last loop

Interestingly, there is fair agreement between the position of the LD kink (t_1, Table I) and the average time when the last hernia is consumed (t_{III}, Table II). This observation allows the shoulder to be interpreted as the stretching and consumption of the many small loops, which with their wide distribution cannot be expected to give rise to any distinct features in the LD average. Only the comparatively coherent death of the last hernia is detected in the spectroscopic ensemble average: first there is as a tendency for the LD to level out when the last loop disappears, and then the average helix orientation increases abruptly as the corresponding amount of chain is transferred to the main arms where it is subjected to more stress and becomes more field aligned. This interpretation of the shoulder is the same as the one given in a simulation version of the present experiment by Long and Viovy [22], who point out that in general many small loops will have lower LD than one correspondingly longer because the DNA at the loop head is of low average orientation [22]. A full comparison with their results, including the effect of molecular weight, will be presented elsewhere [20].

The fact that consistent interpretation of the phases of the LD response can be made by comparing with the *average* times of loop growth and competition from the microscopy data shows that loop dynamics can be studied by use of the LD response in terms of the characteristic times of the oscillations. The amplitude of the LD oscillations, on the other hand, varies with gel concentration in a way that probably can be understood only through a systematic study of the effect of pore size on the detailed shape of the *distribution* of the characteristic times of the molecular reorientation process, as is the case during migration in a constant field [16]. Here we have only determined the mean values and the oscillation amplitudes are therefore not further analyzed. It is worth noticing, however, that an oscillatory LD response in nongelled agarose (0.2% in Fig. 8) is not surprising since the oscillatory mode of migration reflected in the LD oscillations [16] exists also in solutions of non-cross-linked polymers [23,24]. The absence at all fields of an initial LD dip in this system (Fig. 8) is employed below when the LD is interpreted, but the characteristic times of the LD response have not been included in the quantitative analysis because the effective pore size of the transient matrix is not known.

The LD dip reflects stretching of the DNA along the old tube

The negative LD before the probe field is applied (Fig. 7) shows that although the DNA molecules are locally relaxed in the tube, a net orientation of the DNA helix preferentially along the direction of the preparatory field (and thus perpendicular to the probe field) remains also after 10 min of field-free conditions. This can occur because a polymer coil (even in its equilibrium state) has a net preferential orientation of its segments with respect to the direction of its end-to-end vector ([25], see also [1]). In the standard initial state employed here, the end-to-end vector has a preferential orientation perpendicular to the probe field, since the tube is still aligned along the direction of the preparatory field.

The LD dip in Fig. 7 (inset) shows that at low field strengths the probe field initially gives rise to an even higher degree of helix orientation along the old field direction, perpendicular to the applied field. This transiently enhanced perpendicular helix orientation cannot be due to an increase in the tube orientation along the old field direction: tube orientation changes by motion of the DNA molecule out of the old tube [26], and new tube segments thus would be oriented preferentially in the new field direction. In view of Eq. (3) the dip in LD therefore must be due to an enhanced degree of stretching (S_{local}) along the old field direction.

The loops that are observed to be pulled out by the probe field (Fig. 5) can only form and grow by eventually borrowing DNA from the tube segments between the loop holes, and these parts of the chain thus become more stretched along the old tube direction. In the loops, on the other hand, the net orientation will be low initially, because when the loops are still short a large fraction of their DNA will reside in the loop head. There the DNA stretching is low due to collisions with gel fibers as the head tries to find new pores to enter [26], and due to the loops being in effect equivalent to tethered DNA, for which the segment orientation in a field decreases drastically towards the ends [27]. An additional effect in our case is that the strength of the probe field is always lower or equal to that of the preparatory field, so the new tube segments will have lower degree of field alignment than the original tube [which according to Eq. (3) reduces the net contribution to the LD for a given degree of DNA stretching S_{local}]. With these three factors in mind it is reasonable that an enhanced stretching of the DNA between the loopholes along a comparatively strongly aligned tube can more than compensate for the opposite LD contributions from the loops. It is thus possible for the probe field to cause the net helix orientation to be even more preferentially along the old field, but only initially since eventually the loops become so long and few that the high DNA orientation in the loop stems dominate the net LD. Consequently, the absence of an LD dip at high fields (Fig. 7, inset) indicates that the loops stretch at such a high rate that the perpendicular stretching cannot be observed in the LD.

The LD dip as a monitor of loop nucleation

According to the present interpretation the LD turning upward is the earliest sign in the LD response of the loops being formed, and we will therefore use t_0 (Fig. 7, inset) to monitor loop initiation rates. In view of the compensatory effects between perpendicular DNA stretching between loopholes and parallel alignment of loops, t_0 cannot be expected to provide absolute rates of loop nucleation (more concrete, according to the present picture the average loop has penetrated its first pore before t_0). However, it should be remembered that such compensating effects also occur as long as the U is not destroyed, and still the LD and microscopy data give a consistent picture throughout the reorientation process. It is therefore reasonable to use the LD-derived t_0 to

study (on a relative basis) the effects of pore size and field strength on the loop nucleation rate. Consistent with this interpretation $t_0=0$ at all fields in the 0.2% agarose solution (Fig. 8), where no permanent pores exist to impede loop formation.

Importantly, the alternative explanation, that the enhanced stretching along the old tube is caused not by transfer of chain to the loops but to the arms driven by the ends of the molecules, should give rise to another field dependence in t_0 than observed (Fig. 9). In this case the rate of perpendicular stretching would be governed by the rate of migration of the heads, and this mode of electrophoretic DNA stretching is known to give rise to a much weaker field dependence in the LD kinetics in general [8]. This is true also in the particular case of DNA coil deformation studied here, since the expected weaker field dependence is observed in the later stages of the loop dynamics (t_1-t_4 in Fig. 9), which are indeed driven by electrophoretic transport of the ends, as shown here by microscopy.

An arm mechanism for the perpendicular stretching is also in disagreement with the time of the occurrence of the LD dip. Figure 6 shows that the loop dynamics is not under total control of the ends, and therefore local loop-loop competition will govern the early stages of the loop evolution, and this is the time range where the LD dip occurs. This can be seen from t_0 being considerably shorter than t_1 also at the fields where the dip is present (Fig. 9). Thus, the perpendicular stretching reaches a maximum when there are many loops left and the ends still have not taken charge of the reorientation.

The arguments given above against an arm-driven mechanism for the perpendicular DNA stretching are partly based on observations at 5.9 V/cm. It is therefore important that check microscopy experiments showed that loops are formed also at the low fields where the LD dip is observed, so the possibility of pure arm-driven reorientation (no loops) at the low but not high fields can be ruled out. In one experiment in 0.5% agarose the first loop appeared after about 15, 5, and less than 0.5 s at 1.2, 1.6, and 2.6 V/cm, respectively, in fair agreement with the LD-based nucleation times (Fig. 10). The resolution limitations in microscopy render quantitative studies of loop initiation uncertain, however. Furthermore, the slow rate of the overall conformation change at these low fields makes quantitative microscopy studies of the loop dynamics forbiddingly time demanding for the appropriate averages to be obtained. The LD approach allows t_0 to be used to monitor the ensemble average of the time required to nucleate the loops, at the expense of not being based on direct observation.

Source of the loop resistance in the matrix

With the LD dip being controlled by loop initiation we can understand why t_0 is considerably more sensitive to the field strength than the other LD response times (t_1-t_4, Fig. 9). Loop nucleation can only occur by forcing a piece of doubled-over DNA through a pore, whereas the other LD times reflect processes (death of last loop, U formation, and slippage) that are controlled by the electrophoretic transport of the ends, where the DNA is not forced to form a loop [28]. In general there will be a free energy associated with forcing the doubled-over DNA between the obstacles of the matrix, and loop formation will therefore be slowed down when the height of the corresponding energy barrier is comparable to the potential energy gained by moving the DNA segments in the loop down field [22].

The heights of the barriers in the gels used here are low in the sense that there is no observable resistance towards loop formation until the field is below typically 1–3 V/cm, depending on pore size (Fig. 10). A first indication of the origin of the barrier can be gained by estimating the cost of loop formation in different possible mechanisms for loop hindrance in a gel. The energy cost for bending DNA of persistence length P into a half circle to fit into a pore of a radius R can be estimated as [29]

$$W_{\text{bend}}=kTP/R. \tag{4}$$

Even in most dense gel used here P/R is only 1.5 if R is taken to be the average pore radius, and $P=500$ Å as for native DNA. (The results in Fig. 11 suggest that P is shorter for YOYO-stained DNA, if anything.) In addition the relevant pore size is probably larger than the average pore, since loops will first be nucleated through pores that are larger than the average, and still there is an effect of gel concentration on the nucleation rate when the average pore is considerably larger than the persistence length. The same case can be made for possible effects of gel elasticity, because if the DNA loops are able to push gel fibers aside, the effective pore size will again be larger than the average pore size. We thus conclude that the enthalpic penalty for resistance towards DNA bending imposed by the agarose matrix probably is not large enough to seriously slow down loop formation even in the absence of a field.

The free energy cost related to the entropic penalty for a polymer to form a loop out of the reputation tube can be estimated from [22]

$$G=kTps_0, \tag{5}$$

where p is the number of blobs in the loop (a blob corresponds to the DNA in a pore) and s_0 is a dimensionless parameter describing the topology of the matrix, and which is of the order of 1. This contribution to the loop barrier can be seen to grow to several kT even before the loops are long enough to be resolvable in the microscope (the resolution and the blob size are about 0.5 and 0.1 μm, respectively). Long and Viovy have estimated the characteristic field required to overcome the entropic barrier to be

$$E_0=\frac{kTs_0^2}{nqa}, \tag{6}$$

where a is the pore size, q the net charge per Kuhn link (of length b), and $n=a^2/b^2$ is the number of Kuhn segments per blob. For fields $E>E_0$ there should be negligible barrier to loop formation, and for $E<0.1E_0$ loops should form very slowly [22]. With the estimated E_0 of 1.9 (assuming $s_0=1$, $a=2P_E=1800$ Å in 1% agarose, $b=2P=1000$ Å and $q=150$ electron charges) this is a fairly good description of the results in Fig. 10, with a rather abrupt increase in t_0 between 1 and 2 V/cm in 1% agarose.

Equation (6) predicts that E_0 should decrease with increasing pore size as $1/a^3$ and such a high sensitivity to pore size does not seem to be present in the experimental data (Fig. 10). Still the rate of loop formation is clearly slowed down by making the pores smaller, and since the estimated DNA bending resistance to loop formation is very low even in the most dense gel it is interesting to make further comparison with the model for an entropic barrier that underlies Eq. (6).

Fitting the nucleation rate to exponential growth

The model of Long and Viovy [22] predicts that the average time τ for the nucleation of loops by a field-driven crossing of the entropy barrier is

$$\tau = \frac{3\pi^3 \zeta a}{2 s_0^2 q E} \exp\left(\frac{k T s_0^2}{n q E a}\right), \tag{7}$$

where E is the field strength and ζ is the friction coefficient per Kuhn segment (for motion in the tube). The field dependence of the nucleation time t_0 at different gel concentrations (Fig. 10) was fitted to

$$t_0 = \frac{A}{E} e^{E_0/E} \tag{8}$$

(see dotted curves in Fig. 10). Figure 13(a) shows how the fitted parameters A and E_0 vary with the pore size. Clearly E_0 does not vary as strongly as $1/P_E^3$, as predicted by Eq. (6) if a is taken to be the average pore diameter. However, the difference in loop nucleation rates in different gel concentrations probably reflects the concentration of larger than average pores, rather than the average pore size. Thus E_0 may be comparatively insensitive to P_E because the concentration of pores around 1000 Å (which gives E_0 values in good agreement with experiment) is less sensitive to gel concentration than the average pore size.

The main effect of an increased gel concentration is to increase the prefactor A, which is proportional to the pore size a [Eq. (7)]. The interpretation of this observation is difficult in view of the uncertainty regarding the relevant value for the a for the loop processes studied here. It is

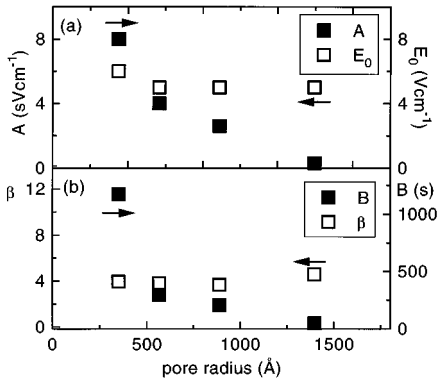

FIG. 13. Parameters of best fits of nucleation time t_0 to Eq. (8) (top, see Fig. 10) or Eq. (9) (bottom, see Fig. 9) vs average pore radius of the gel. *G*-DNA.

expected, however, that a should decrease with increasing gel concentration, and hence there must be an opposing factor that explains how A can increase with increasing gel concentration. The prefactor A is proportional also to the friction factor ζ, which indeed can be expected to increase with increasing gel concentration, but it is not known if the dependence is as strong as suggested by the over 200-fold increase in A going from 0.5% agarose to 2% nusieve. Clearly, it would be desirable to test the notion of an entropic barrier to DNA-loop formation in a more well-defined lattice.

For comparison Fig. 13(b) shows the values of the best fits to a power-law dependence for t_0:

$$t_0 = B E^{-\beta} \tag{9}$$

(see Fig. 9), which in fact gave somewhat better fits than Eq. (8). The power-law exponent (β) is insensitive to a decrease in gel concentration, which mainly has the effect to decrease the prefactor as strongly as was observed with Eq. (8).

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